LABORATORY NOTEBOOK

Notebook No.: _____

Assigned to: ARTHUR BRANSTROM

Date: ___/___/___

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Exhibit 9

Rev. 1984

Construction of new plasmid pAB103
pMB1 ORI of replication
pSC101 ORI of replication
ASD (BglII Fragment) FROM pYA3167
PLASMID pAB102 WAS DIGESTED W BglII to remove E. coli ASD
& replaced W pYA3167 ASD.
Ligated DNA was transformed into X6097 cells to isolate plasmids of interest.
Km^R cassette was inserted @ the BAMH1 restriction site to check for in vitro plasmid stability.
1321 [OVERNIGHT GROWTH IN LB ~~W~~ AMP]
@ 12 HR time point, 96/100 Km^R
@ 50 GENERATIONS (6 HRS IN FRESH Km^R MEDIA) 95/100 Km^R
2000 [OVERNIGHT CULTURES GROWN IN LB AMP]
@ 12 HRS 67/100 Km^R
@ 50 GENERATIONS 55/100 Km^R
1321 ASD containing plasmids pAB102 or pAB103 were given to D. Szymanski to pass thru mice (BALB/c - only)
- HIV genes VIF & GAG are cloned into the BAMH1 & SAL1 sites of plasmid pAB103 (under control of glac)
Expression was detected for both by using rabbit sera or human sera respectively.
- The VIF & GAG constructs were given to D. Szymanski to pass thru the mice again.
While recovery varied from that observed with pAB102 constructs (i.e. lower recovery of bacteria from the spleens, appears to be slower in vivo growth rate), isolates were collected that expressed levels of VIF & GAG that were as high as what went in. ORF VIF was overexpressed in pAB102 isolated from spleens. Plasmids from low expressing & high expressing strains are isolated, & will be used to complement back plasmid cured strains (HIGH & LOW level expressers) to determine if the mutation in vivo is occurring @ the plasmid or chromosomal level

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2 different regions of the ENV gene are PCR amplified
- the regions chosen are conserved for the primer sequences between MN & III_B.
- Amplification from both HIV genomes amplified similar size fragments. Sequences were pulled from the GCG database.
- THESE 4 PCR products are BAMHI/SALI digested & cloned into pUC18 (β-gal indicator + overexpression to determine if the envelope constructs are being translated)

Arthur A Brandstadter

NOTE: TEXT PRINTED FOR FUTURE "READABILITY"

→ 2 ENV REGIONS WERE ISOLATED, CLONED INTO pUC18:
- ENTIRE gp160 (FROM BOTH MN & III_B)
- V_3 LOOP REGION (FROM BTH MN & III_B)
  III_B ENCODES A.A. FROM * 263-610
- V_3 LOOP CONTAINING CLONES DESIGNATED ENV_{MN} A1 OR ENV_{III_B} /

THE ENTIRE GP160 APPEARS TO BE POORLY EXPRESSED COMPARED TO THE A1 REGION.
- EXPRESSION WAS DETECTED BY HUMAN SERA

Arthur A. Brandstadter

ANALYSIS OF 1321 ΔASD, PRIOR CONSTRUCTS THAN MICE AFTER COLLECTION OF BACTERIA FROM MOUSE SPLEENS, KIF OR GAG EXPRESSION WAS ANALYZED WITH KIF ANTI-SERA (RA333.F), OR HUMAN SERA TO GAG. EXPRESSION WAS VARIABLE FOR BOTH ISOLATES, SOME BEING LOW LEVEL EXPRESSORS, AND SOME BEING COMPARABLE TO WILD-TYPE. IT APPEARS THAT THE DECREASED LEVELS OF EXPRESSION ARE DUE TO REDUCED PLASMID COPY NUMBER. ASD PRIMERS WERE REMADE TO AMPLIFY ASD & ITS ENDOGENOUS PROMOTER, & CLONED UNDER THE CONTROL OF p Rac. p Rac DID NOT RESULT IN A DECREASE IN ASD PRODUCTION.

AAB

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pAGC WAS CLONED INTO pAB103 RESULTING IN PLASMID DESIGNATED pAB104. pAGC WAS CLONED VIA BglII & SALI RESTRICTION SITES, & THE REINTRODUCTION OF ASD (FLANKED BY BglII SITES). THIS CLONE CONTAINS: pSC101 ORIGIN OF REPLICATION, pUC18 ORIGIN OF REPLICATION, ROY CURTISS'S ASD FROM S. Typhimurium, pAGC PROMOTOR & BAMHI - SALI POLYLINKER

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COMPLETE ENV (GP160) REGIONS FROM BOTH HIV$_{MN}$ & HIV$_{BRU}$ ARE CLONED INTO THE VARIOUS DELIVERY VECTORS. pAB102 & pAB103 ARE USED TO EXPRESS THE SEQUENCES. ALTHOUGH DETECTION WAS NEGLIGIBLE DUE ERRATUM TO THE LOWER COPY #'s OF THESE PLASMIDS COMPARED TO pUC18, OR TO THE INSTABILITY OF THE gp160 SEQUENCE

Arthur A. Brandt

IN COLLABORATION = D. SIZEMORE, THE VIF WAS CLONED INTO PLASMID pAB104 & ASSAYED FOR EXPRESSION IN E. coli JM109, EXPRESSION IS = EXPRESSION FROM pAB102 OR pAB103 (plac PROMOTER).

Arthur A. Brandt

THE ANTIGEN RECOGNITION FRAGMENT "TNRNPQ" WAS CLONED (AFTER SYNTHESIZING) INTO VARIOUS VECTOR CONSTRUCTS. COMPLEMENTARY SEQUENCES: T N R N P Q

FORWARD OLIGO GATC GT ACG AAC CGG AAT CCT CAG

REVERSE OLIGO CATGC TTG GCC TTA GGA GTC CTAG

MUTATED BAMH1 SITE — CONSENSUS BAMH1 SEQUENCE

THE DIFFICULTY IN DETECTING POSITIVE CLONES WAS CIRCUMVENTED BY CONSTRUCTING TNRNPQ SEQUENCES TO BE DIRECTIONALLY CLONED USING ECOR1 & BAMH1 RESTRICTION SITES. THE OLIGO WAS DESIGNED TO BE PLACED ON THE AMINO-TERMINAL END OF THE EXPRESSED PROTEIN.

FORWARD OLIGO AATT GT GGT ACG AAC CGG AAT CCT CAG GGG

REVERSE OLIGO CA CCA TGC TTG GCC TTA GGA GTC CCCC TAG

THESE OLIGO's ANNEALED & LIGATED INTO pAB18::VIF @ THE BAMH1/ECOR1 SITES. EXPRESSION VIA 2A10 ANTIBODY WAS REACTIVELY NON-EXISTANT ALTHOUGH VIF WAS BEING TRANSLATED DUE TO ITS REACTIVITY @ VIF ANTISERUM.

Arthur A. Brandt

THE IMPORTANCE OF HAVING TNRNPQ ON THE NH$_2$ OR COOH TERMINAL END WAS EVALUATED USING VIF-TNRNPQ ENGINEERED SEQUENCES. THESE C.(VIF-TNRNPQ) CONSTRUCTS WERE VERY POORLY RECOGNIZED BY THE 2A10 ANTIBODY. THE PROBLEM APPEARS TO BE THE RESULT OF NOT ENOUGH COPIES OF THE TNRNPQ SEQUENCE FOR THE 2A10 ANTIBODY TO RECOGNIZE.

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A TAQMAN PROBE OF TWNPQ IS SYNTHESIZED AS A VIF FUSION OLIGO (127 bp) TO BE USED IN PCR AMPLIFICATION OF VIF F PROTEIN. THIS RESULTED IN A VIF-$[TWNPQ]_4$ FUSION, WHICH WAS PCR DERIVED & CLONED INTO BAM/SAL SITES OF pAB102 & pAB104. EXPRESSION WAS DETECTABLE, BUT NOT AS REACTIVE WHEN COMPARED TO VIF REACTED WITH RABBIT anti-VIF SERUM.

Nathan A. Brandon

THE $[TWNPQ]_4$ SEQUENCE IS USED TO TAG ENV EXPRESSION IN VARIOUS PLASMID CONSTRUCTS TO DETERMINE IF DIFFICULTY IN DETECTION IS DUE TO POOR EXPRESSION OR THE LACK OF HIGH AFFINITY/TITER ANTIBODY.

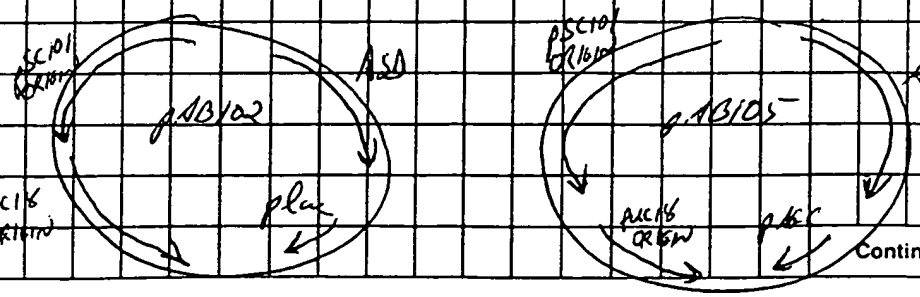
ENV_{MN} & ENV_{IIIB} ARE CLONED FROM BOTH HIV_{MN} & HIV_{IIIB} UTILIZING CONSERVED SEQUENCES (ENTIRE GP160 TAGGED @ BEGIN END OF PROTEIN). PCR AMPLIFIED DNA IS CLONED INTO BAMHI/SALI SITE OF pAB101, pAB102, ~~pAB103~~. ENV_{IIIB} CONSTRUCTS WERE MORE UNSTABLE THAN ENV_{MN} CONSTRUCTS, RESULTING IN LARGE DELETIONS OF THE PLASMIDS. NEITHER SET OF CONSTRUCTS EXPRESSED PROTEIN DETECTABLE BY 2A10 ANTIBODY.

Nathan A. Brandon

ASD FROM pAB102 WAS INTRODUCED INTO A PLASMID (pAB104) CONTAINING THE pUC ORIGIN OF REPLICATION. THIS E. coli ASD (DglIX) HAD THE POTENTIAL OF BEING IN 2 POSSIBLE ORIENTATIONS. DOUBLE DIGESTS WITH BAMHI & HINDIII DETERMINED THAT ASD IN pAB105 IS IN THE SAME ORIENTATION AS [illegible]

Nathan A. Brandon

THE ORIENTATION OF THE pUC18 ORIGIN OF REPLICATION WAS DETERMINED BY PCR ANALYSIS. THE ORIENTATION OF REPLICATION CONVERGES TOWARDS THE PROMOTER (plac IN pAB102 OR pTAC IN pAB105)



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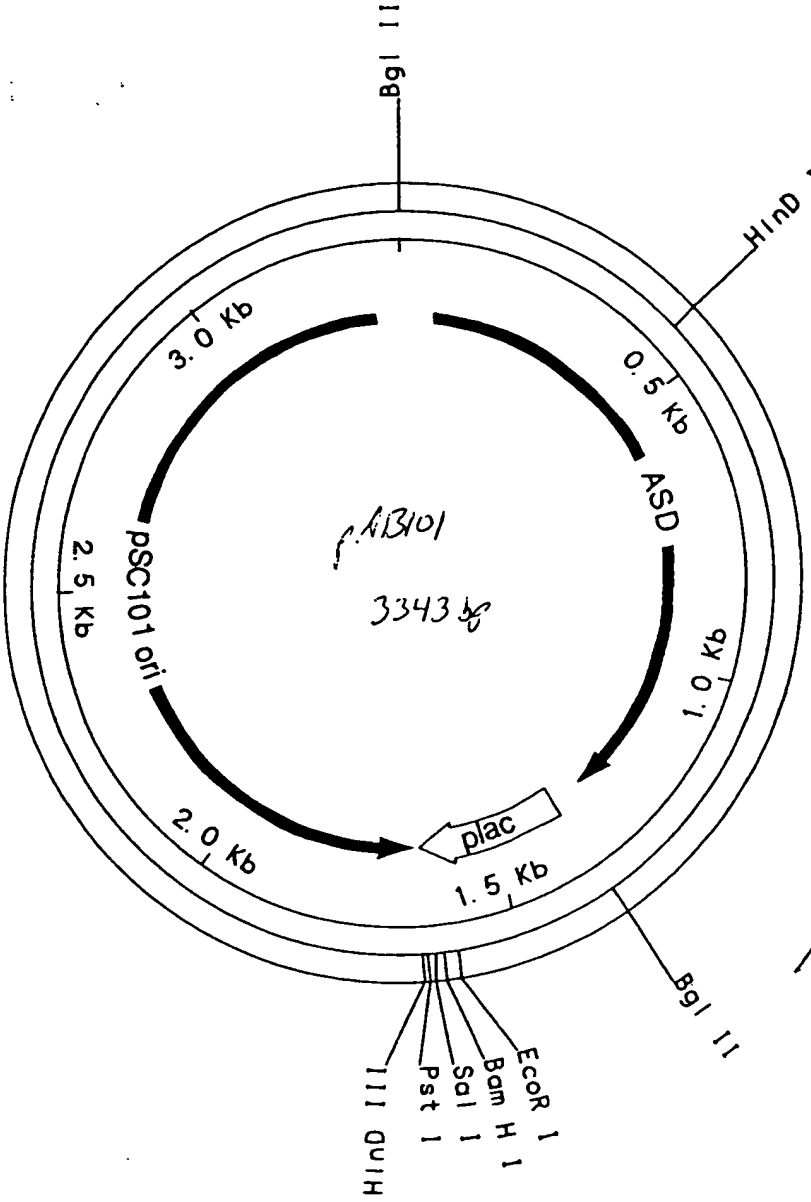
PLASMID MAPS ARE CONSTRUCTED FROM THE GCG DATA BANK, AND ARE MADE FOR ALL 5 PLASMID CONSTRUCTS, pAB101 → pAB105, WITH SUBTLE DIFFERENCES BETWEEN VARIOUS SEGMENTS.



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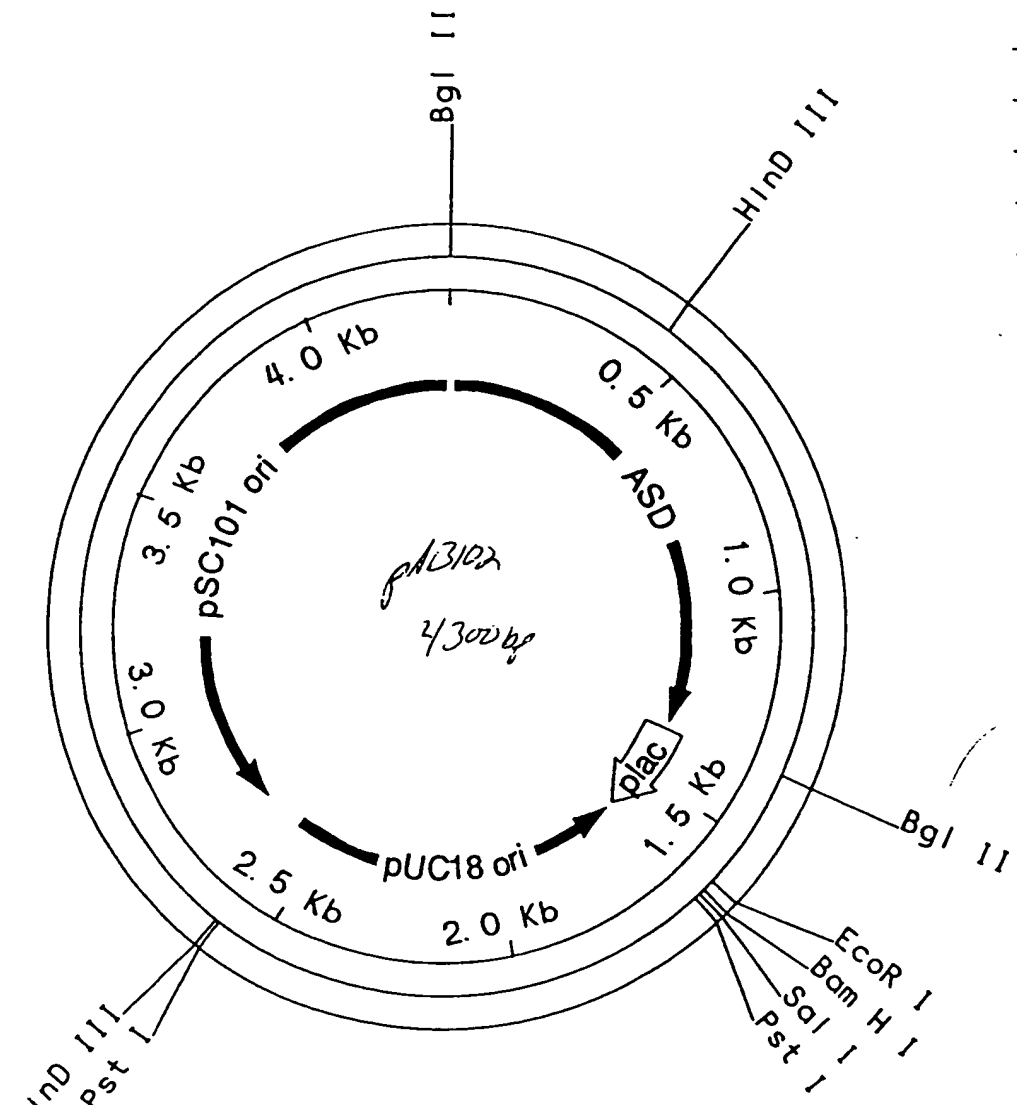

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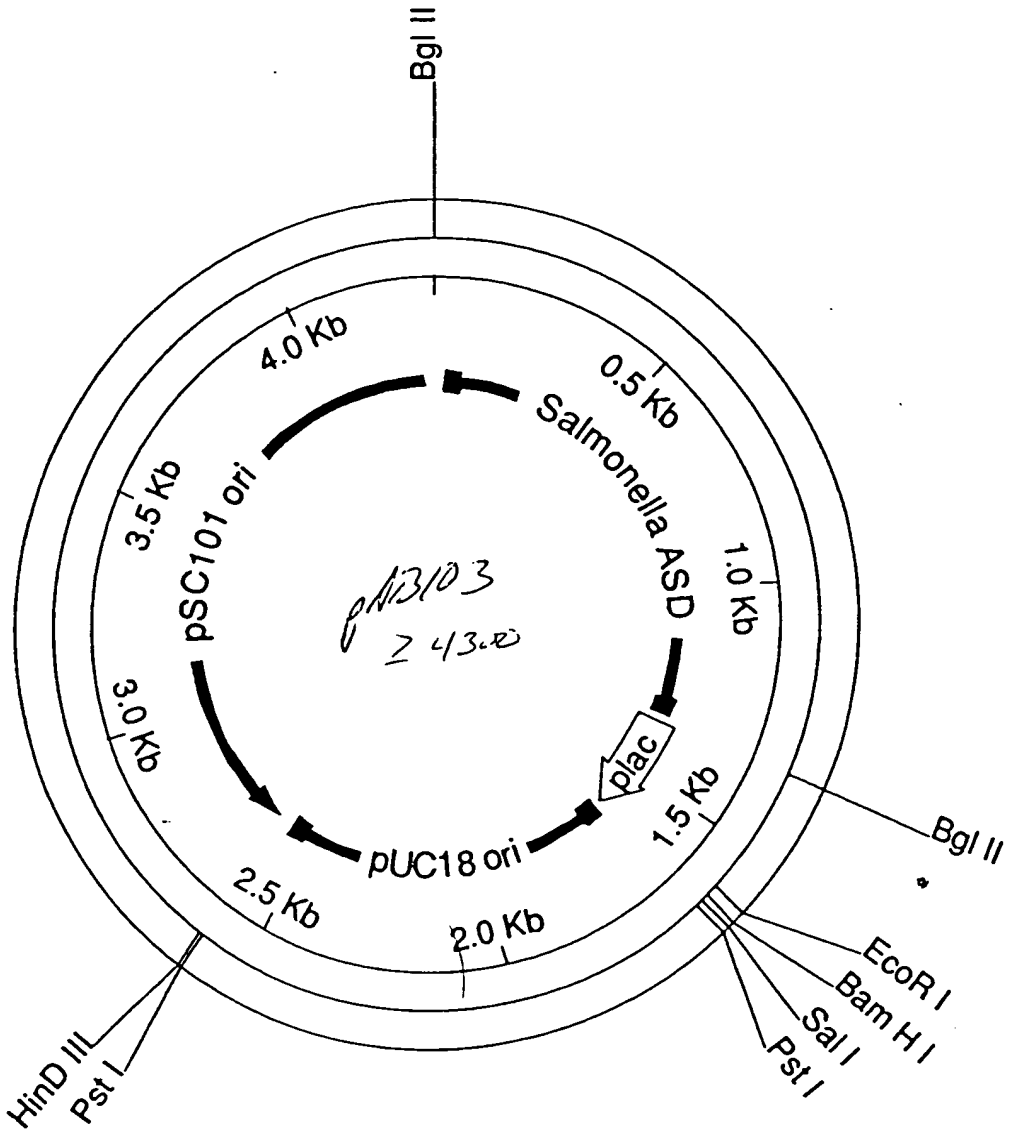

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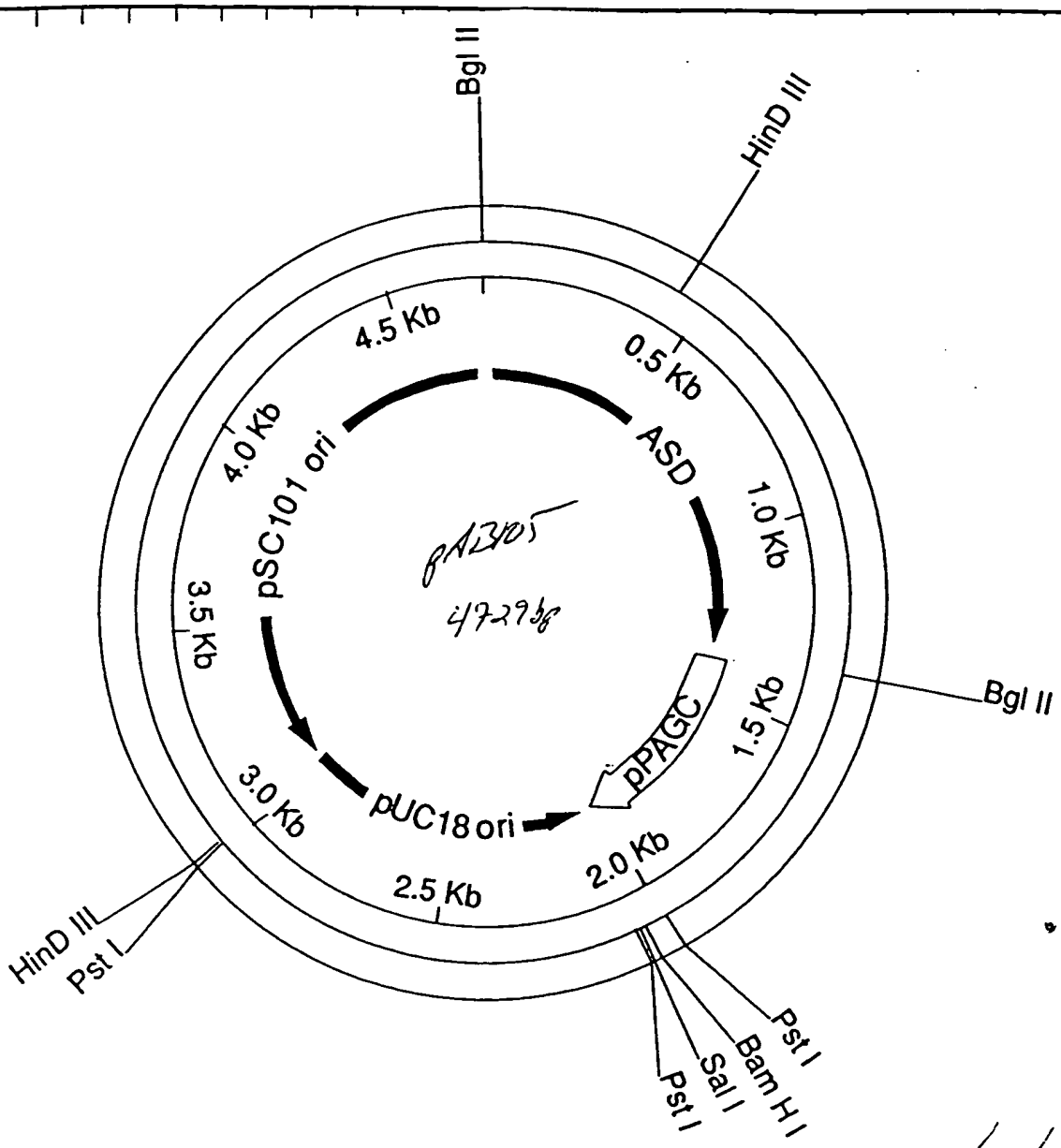

pAZ105
47298

Nathan A Brandstrom

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SEQUENCING - A GENE FRAGMENT WAS CONSTRUCTED FOR VIF USING PLASMID pRIT2T (PHARMACIA). VIF PRIMERS WERE MADE TO ALLOW IN FRAME CLONING WITH OMNI SITE, & UTILIZATION OF SALI SITE DOWNSTREAM OF VIF FOR DIRECTIONAL CLONING.

Nathan A. Brandon

(TERRI GONZALEZ)

CHROMOSOMAL PROBES WORK DONE FOR 1321 ALSO CONSTRUCTED FOR ISOLATES CONTAINING PLASMID pAB101 → pAB105. THE PLASMID COPY #'S WERE COMPARED TO S. typhimurium 1321 RECOVERED FROM MOUSE SPLEENS THAT CONTAINED pAB103::GAG THAT WAS EXPRESSED POORLY, & pAB103::VIF THAT WAS ALSO WEAKLY EXPRESSED AS COMPARED TO WILD-TYPE BEFORE PASSAGE THRU MICE.

Nathan A. Brandon

THE VIRAL DNA SEQUENCE CONTAINS A PUTATIVE PROTEOLYTIC CLEAVAGE SITE AT POSITION #7774/bp. IF THIS SITE WAS RECOGNIZED IN E. coli OR SALMONELLA (OR SHIGELLA), THE EXPECTED PRODUCTS WOULD BE 27,805 Kdal (7040 - 7774/aa) AND 11,182 Kdal (7775 - 8083 aa). THIS DOES NOT APPEAR TO BE THE CASE.

Nathan A. Brandon

ELECTROPORATION FOR S. FLEXNERI 2457T WAS ASSAYED, & FOUND TO BE OPTIMAL FOR THE FOLLOWING CONDITIONS:

2.0 KVOLTS, 25 uF, 800 Ω. CELLS WERE PREPARED VIA THE PROCEDURE FOR "PREPARATION OF E. coli CELLS FOR ELECTROPORATION." USING THIS PROCEDURE, PLASMID pUC19::HIV III WAS INTRODUCED INTO VIRULENT 2457T. PUC19 CONTAINED GENE PREVIOUSLY DETERMINED TO BE STABLE, AND HAD NO ADVERSE EFFECTS ON THE VIRULENCE PLASMID. THIS STRAIN WAS TESTED FOR INVASIVENESS BY A PLAQUE ASSAY DONE BY ED OAKS, AND THE STRAIN WAS ISOLATED FROM A PLAQUE. THIS ISOLATE WILL BE TESTED FOR AN IMMUNE RESPONSE IN THE MOUSE-LUNG MODEL. STRAINS #402 & M4:43 A1 WERE ALSO TRANSFORMED

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6x His primers were synthesized for use in protein purification of cloned genes. Purification is obtained using Ni^{+2} binding resin (Qiagen)

6x His F 5' GATC/AT CAC CAT CAC CAT CAC GGC

6x His R 3' TA GTG GTA GTG GTA GTG CCC CTAG

Mutated BamHI restriction site. Allows cloning to occur downstream of the 6x His tag.

VIF was cloned/expressed in this system, but some protein contamination still occurs upon elution from the Ni^{+2} column.

Arthur A. Brandstadter

Suicide vectors were finally constructed in which ASD can be mutated in the chromosome of virtually any Gram negative bacteria having sequence homology with E. coli ASD. The smallest construct is the result of a PCR amplification (reverse) of ASD removing 552 bp from the internal sequence of ASD, rendering it non functional. Into this region, the SmaI Km^r cassette is ligated. The entire ASD::Km^r region was PCR amplified with SalI & SacI restriction sites for cloning into the suicide vector pCVD442.

Arthur A. Brandstadter

AAB

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A SHIGELLA SONNEI STRAIN OBTAINED FROM MARK HEATH IS ATTENUATED FOR INVASION (INVA⁻2). UNFORTUNATELY, THIS STRAIN STILL CONTAINS THE Amp^R REGION, SO A MANIPULATION OF MOBILIZATION VECTOR CANNOT BE DIRECTLY INTRODUCED INTO THIS STRAIN.

THE CONCEPT BEHIND THAT SHIGELLA COULD SERVE AS A CARRIER OF FOREIGN DNA, MUCH LIKE A VIRUS DNA DELIVERY SYSTEM. THE Amp^R PLASMID pCMV3 WAS OBTAINED FROM CLONTECH. 2 PLASMID WORK CONSTRUCTIONS:

→ pBR322 #24 WAS CONSTRUCTED BY PST I/EcoR I DIGESTION TO DESTROY Amp^R, & RELIGATION WITH A PST I/EcoR I POLYLINKER. THIS PLASMID WILL BE USED TO TAG RECIPIENTS = Tc^R FOR SUICIDE VECTOR MATINGS.

→ pBR322 #[illegible] : pCMV3 #5A WAS CONSTRUCTED BY PST I DIGESTIONS & SUBSEQUENT LIGATION OF THE 2 VECTORS. THIS CONSTRUCT CAN NOW BE USED IN Amp^R BACKGROUNDS THAT ARE Tc^S

Arthur A. Brandon

CONJUGATION EXPERIMENTS WITH DH5α CONTAINING THE SUICIDE VECTOR pCVD442 : 0.85 / Km^R WERE UNSUCCESSFUL. CONJUGATIONS WERE SUCCESSFULLY PERFORMED WHEN THE PLASMID WAS PLACED INTO SM10λpir & MATED WITH VIRULENT 24577 CONTAINING THE Tc^R PLASMID pBR322 #2A. PCR SCREENING WAS NECESSARY TO DETECT FALSE INTEGRATIONS AS OPPOSED TO PLASMID MAINTENANCE & INTEGRATION INTO THE WRONG SITES.

Arthur A. Brandon

SUBSEQUENT PCR ANALYSIS DETERMINED THAT AT LEAST 3 CLONES WERE CORRECT, & CHECKED OUT FOR VIRULENCE PLASMID MARKERS Km^R COLONIES ARE PLATED ONTO SUCROSE NA PLATES AND SCREENED FOR Amp^R, OAP REQUIREMENT, & CR BINDING & Tc^R. 2 CLONES ARE PICKED THAT HAVE ALL THE PROPER PHENOTYPES & GENOTYPES, 24577, Δ322, ΔAP5::Km^R #15 & #19A

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LARGE SCALE PURIFICATION OF VIF WAS ATTEMPTED USING GENES RESULTED IN PROTEIN COLLECTED PREDOMINANTLY VIF, BUT OTHER BANDS AS WELL, INCLUDING WHAT APPEARS TO BE LIKELY THAT ELECTROPHORESED AT THE SAME MOLECULAR WEIGHT. ELUTION CONDITIONS WERE NOT OPTIMIZED, & THE PROTEIN WAS ELUTED C 50mL BUFFER C (GIAGEN) CONTAINING 250 mM IMIDAZOLE.

Arthur A. Brandstadter

GROWTH CURVES ARE PERFORMED ON STRAINS 2457T, D322 #15c (ALSO), 2457T WILD-TYPE, & MUTANT STRAINS #15c & #19A COMPLEMENTED WITH pA302. WHILE OD's ARE VERY COMPARABLE, THE # OF VIABLE BACTERIA FOR TIME POINT VARIED CONSIDERABLY. IT WAS ALSO DETERMINED THAT #15c GROWN IN THE ABSENCE OF DAP HAD A 10-FOLD LOSS OF VIABILITY EVERY 30 MINUTES FOR THE FIRST COUPLE OF HOURS WHEN THE CELLS HAD BEEN GROWING LOGARITHMICALLY. GUINEA PIGS INFECTED WITH #15c DID NOT FORM CONJUGATES (J. HARTMAN) AND THE COMPLEMENTED STRAIN DID, THOUGH NOT TO THE EXTENT OF WILD-TYPE 2457T.

Arthur A. Brandstadter

TO TEST THE POSSIBILITY OF β-GAL EXPRESSION FROM pCNB WITHIN CELL LINES VIA DONORY BY #15c SHEDDING, THE PLASMID IS INTRODUCED VIA ELECTROPORATION. IT APPEARED THAT THE STRAIN #15c WAS VERY RESISTANT TO TRANSFORMATION - DUE POSSIBLY TO THE pBR322 BASED PLASMID ALREADY THERE, AND THE WEAKENED STATE OF THE DAP REQUIRING CELLS

Arthur A. Brandstadter

A Thy A⁻3 CONSTRUCT FROM W. Harris WAS OBTAINED TO DETERMINE IF IT COULD SERVE AS A DELIVERY VEHICLE FOR pCNB TO BHK CELLS. UNFORTUNATELY THIS STRAIN WAS FOUND TO BE CR⁻ & THEREFORE WOULDN'T INVADE. HANK IS ATTEMPTING TO RECONSTRUCT THE STRAIN.

Arthur A. Brandstadter

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ENVIII GENE WAS CLONED INTO PLASMID pJW4303 (MULLINS PLASMID CONTAINING TPA LEADER SEQUENCE). THIS PLASMID WAS OBTAINED FROM JOFF LYON. WE CANNOT USE THIS PLASMID WITHOUT OBTAINING PERMISSION FROM AGRACETUS FIRST. THIS CONSTRUCT HAS YET TO BE TESTED FOR EXPRESSION IN MAMMALIAN CELLS.

Arthur A. Brantly 4/3/95

THE NITROMYCIN GENE WAS CLONED FOR USE IN SELECTING DNA DELIVERY VECTORS IN VITRO. THE GENE WAS CLONED INTO pUC19 NINDIII/SALI CLONED INTO pCMVβ. IT HAS BEEN DETERMINED THAT THE NYG CONCENTRATION FOR DHFR OR PBLS CELL LINES MAY NEED TO BE TITERED FOR EACH CELL LINE, 4 2-FOLD DILUTIONS WILL BE TESTED (200-800 μg/ml?).

Arthur A. Brantly

A WHITE ISOLATE OF ISO, pCMVβ WAS PICKED FROM A HEAVY STREAK OF COLI ON A TSA, X GAL AMP 200 PLATE. THIS ISOLATE WAS FOUND TO BE lacZ (β-GAL ANTIBODY) & lacC (β-GAL ANTIBODY) NEGATIVE. THIS STRAIN IS TO SERVE AS THE CONTROL IN THE INVASION ASSAY TO SHOW THAT UPTAKE & SUBSEQUENT EXPRESSION OF β-GAL IS DUE TO THE INVASIVE PHENOTYPE OF ISO.

Arthur A Brantly

THE TAT-ROV GENE SEQUENCE FROM HIV-1 IS CURRENTLY BEING AMPLIFIED & CLONED INTO A DNA EXPRESSION VECTOR (pCMVβ). WHILE AMPLIFIED PRODUCT CAN BE OBTAINED, THE PRODUCT REFUSES TO BE CLONED. VARIOUS RESTRICTION SITES HAVE BEEN INCORPORATED INCLUDING HIND III, NHE I, KPN I, CLA I. AFTER SUB-CLONING INTO A VECTOR SUCH AS pUC18 IT APPEARS THAT THE 3' END RESTRICTION SITE IS MISSING, & THE PRIMERS APPEARS TO BE FULL LENGTH.

Arthur A Brantly

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FOR CONSTRUCTION OF VARIOUS INSERTS INTO THE pCMV3 BACKBONE, THE VECTOR WAS DIGESTED WITH NOT I, AND PURIFIED & LIGATED BACK TO ITSELF. THIS pCMV3 NOT I VECTOR WILL BE USED FOR SUBSEQUENT CLONING INTO NOT I SITE. PCR AMPLIFICATION OF THE VECTOR TO ELIMINATE UNIQUE RESTRICTION SITES WAS NOT NEEDED FOR THE SUBSEQUENT CLONING OF INSERTS.

Nathan A. Brandt 6/20/95

THE ASD WILL BE SEQUENCED FROM BOTH 5 FRAGMENT & 3 SOURCE FOR FUTURE USE IN MAKING ASD MUTATIONS & TO DETERMINE IF THE 5 SOURCE PROBLEMS IN GENERATING AN ASD MUTANT IS RELATED TO SEQUENCING DISCREPANCIES.

Nathan A. Brandt 7/7/95

GUINEA PIG EXPTS ARE BEING REQUIRED FOR THE 3rd TIME TO DETERMINE IF 15d & 15g pCMV3 CAN PROTECT. PRELIMINARY RESULTS ARE POSITIVE, ALTHOUGH IT APPEARS THAT 15g IS MORE (SLIGHTLY - THOUGH) PROTECTIVE THAN 15d pCMV3.

Nathan A. Brandt 7/25/95

THE DETERMINATION OF COPY NUMBER OF THE SUMMARIZED ASD PLASMIDS WILL BE PERFORMED USING A COMBINATION OF PCR REACTIONS UTILIZING PRIMERS FOR ASD, THE ORIGIN OF REPLICATION FOR 101, & SS4. THIS WILL DETERMINE COPY NUMBERS FOR pASD, 102, 103, 104, & 105. COPY # CAN ALSO BE DETERMINED FOR THOSE PLASMIDS CONTAINING INSERTS TO DETERMINE WHAT EFFECTS THOSE INSERTS HAVE ON COPY NUMBER.

Nathan A. Brandt 8/11/95

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